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# Dimerization of Transcobalamin II Receptor

## REQUIREMENT OF A STRUCTURALLY ORDERED LIPID BILAYER*

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**Transcobalamin II receptor (TC II-R) exists as a monomer and a dimer of molecular masses of 62 and 124 kDa in the microsomal and plasma membranes, respectively, and *in vitro*, pure TC II-R monomer dimerizes upon insertion into egg PC/cholesterol (molar ratio, 4:1) liposomes (Bose, S., Seetharam, S., and Seetharam, B. (1995) *J. Biol. Chem.* 270, 8152–8157 and Bose, S., Seetharam, S., Hammond, T., and Seetharam, B. (1995) *Biochem. J.* 310, 923–929). The current studies were carried out to define the mechanism of TC II-R dimerization. Both the mature TC II-R (62 kDa) and the enzymatically deglycosylated TC II-R (45–47 kDa) demonstrated optimal association and formed dimers of molecular masses of 95 and 124 kDa, respectively, at 22 °C when bound to egg PC vesicles containing at least 10 mol % of cholesterol. Mature TC II-R dimerized upon insertion into synthetic phosphatidylcholine vesicles of different fatty acyl chain length (dimyristoyl, dipalmitoyl, and disteroyl phosphatidylcholine) in the absence or the presence of cholesterol at temperatures below or above their transition temperatures, respectively. Dimerization of TC II-R also occurred with vesicles prepared using lipid extract from the plasma but not microsomal membranes. Cholesterol depletion of native intestinal plasma membranes or its enrichment in the microsomal membranes resulted in the *in situ* conversion of the 124-kDa dimer to the 62-kDa monomer or of the monomer into the dimer form, respectively. Treatment of plasma membranes with phospholipase $A_2$ resulted in the conversion of the dimer form of the receptor to the monomer form and spin label studies using 1-palmitoyl, 12 doxylsteroyl phosphatidylcholine revealed that interactions of TC II-R with PC vesicles increased order around the probe. Based on these results we suggest that dimerization of TC II-R is mediated by its interactions with a rigid more ordered lipid bilayer membrane, is regulated in plasma membranes by cholesterol levels, and is independent of glycosylation-mediated folding.**

Plasma transport of absorbed Cobalamin (Cbl; vitamin $B_{12}$)[1] to tissues is mediated by transcobalamin II (TC II), a 43–45-kDa nonglycoprotein plasma Cbl binder (1). The tissue/cellular uptake of TC II-Cbl occurs via receptor-mediated endocytosis (2) and is mediated via TC II-R expressed in the plasma membranes of all tissues (3–5). The importance of plasma membrane expression of TC II-R in plasma transport of Cbl is borne out by our recent observation (6) that *in vivo*, functional inactivation of TC II-R by its circulating antibody results in the development of Cbl deficiency. Recent studies (4) from our laboratory have shown that TC II-R exists as a dimer of molecular mass of 124 kDa in all tissue membranes and that the dimerization of TC II-R is due to noncovalent interaction between two monomers of molecular mass of 62 kDa and *in vitro* is mediated by its interactions with lipid bilayer prepared using egg PC and cholesterol. Our recent immunoblot studies (4–6) have identified TC II-R dimers in the tissue membranes across species, and in the rat kidney, the 62 kDa monomer was present only in the microsomes, whereas the 124-kDa dimer form of the receptor was the only form of TC II-R present in the apical and basolateral membranes, intermicrovillar clefts, and clathrin-coated vesicles. Neither the monomer nor the dimer form of the receptor was detected in the light endosomes and lysosomes (5). The steady state level of TC II-R dimer is 8–10-fold higher than that of TC II-R monomer in all the tissue membranes tested (5). Taken together, these studies (4, 5) have indicated that dimerization of TC II-R is a rapid, post-microsomal event and that the inability of the TC II-R to dimerize in the microsomal membranes could be due to (*a*) lack of lipid microenvironment essential for its dimerization, (*b*) improper folding of TC II-R due to incomplete maturation of its *N*- and/or *O*-linked sugars, or (*c*) weaker interaction between the two monomers such that the treatment with SDS results in the disassociation of the dimer.

The present investigation was carried out to further explore the mechanism of TC II-R dimerization and to examine which of the abovementioned possibilities are operational in mediating the noncovalent interaction between the two monomers of TC II-R. The results of the current study show that (*a*) *in vitro*, the dimerization of TC II-R is regulated by a rigid, more ordered lipid bilayer, (*b*) *in vivo*, higher cholesterol levels of plasma membranes provide a more ordered lipid microenvironment to facilitate the dimerization of TC II-R, and (*c*) the dimerization of TC II-R is not influenced by folding alterations due to its *N*- and *O*-glycosylation.

## MATERIALS AND METHODS

The following chemicals were purchased as indicated: egg PC, dimyristoyl PC, dipalmitoyl PC, disteroyl PC, 1-palmitoyl 12-doxylsteroyl-*sn*-glycero-3-phosphocholine, and cholesterol (Avanti Polar Lipids Inc. Alabaster, AL); [$^{57}$Co] cyanocobalamin (15 $\mu$Ci/$\mu$g) and carrier-free $Na^{2+}$ $^{125}$I (Amersham Corp.); peptide *N*-glycosidase from *Flavobacterium menengosepticum* and *O*-glycosidase from *Diplococcus pneumoniae* (Boehringer Mannheim); phospholipase $A_2$ from bee venom, phospholipase C from *Clostridium Welchi*, phospholipase D from peanut, sialidase from

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[1] The abbreviations used are: Cbl, cobalamin (vitamin $B_{12}$); TC II, transcobalamin II; TC II-R, transcobalamin II receptor; PC, phosphatidylcholine; DMPC, dimyristoyl PC; DPPC, dipalmitoyl PC; DSPC, disteroyl PC; PAGE, polyacrylamide gel electrophoresis; BLM, basolateral membranes; EPR, electron paramagnetic resonance.



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*Clostridium Perifringens*, dihydrocholesterol, 7-keto cholesterol, and protein A (Sigma).

Pure TC II-R was obtained from human placenta essentially as described earlier (4). Monospecific antiserum to TC II-R was prepared in rabbits as described earlier (4). The ligand TC II used in the TC II-R assays was partially purified from human plasma according to Lindemans *et al.* (7). TC II-R activity in the lipid vesicles was determined using human TC II-[$^{57}$Co]Cbl (2 pmol) and the Triton X-100 extracts of the reconstituted vesicles by the DEAE-Sephadex method of Seligman and Allen (8).

*Isolation of Rat Intestinal Microsomal and Basolateral Membranes*—Rat intestinal mucosal microsomes were prepared from a 5% (w/v) homogenate of intestinal mucosa prepared in 0.25 M sucrose containing 5 mM EDTA and 10 mM sodium phosphate buffer, pH 7.4. The post-mitochondrial supernatant was centrifuged at 150,000 × *g* for 2 h, and the pellet obtained was washed in the same buffer once and re-pelleted membranes were used as microsomes. The microsomal membrane contained less than 0.5–1% of the apical and basolateral markers, alkaline phosphatase, and $Na^+/K^+$-ATPase, respectively. The basolateral membranes were prepared using differential and sorbitol gradient centrifugation according to Walters *et al.* (9). These membranes were enriched 12-fold for the marker $Na^+/K^+$-ATPase with a recovery of 14% and contained <1–2% of the apical markers, alkaline phosphatase, and γ glutamyl transpeptidase.

*Lipid Extraction and Modulation of Lipid Composition of Microsomal and Basolateral Membranes*—Microsomal or basolateral membranes (5–10 mg of protein) in 2 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride was treated with 16 ml of chloroform-methanol (2:1), vortexed, and allowed to stand for 30 min. The mixture was centrifuged for 10 min at 3000 rpm, and the organic layer was removed for total phospholipid and cholesterol estimations. Total phospholipid was estimated following hydrolysis of the nitrogen dried lipid extract with 70% perchloric acid and estimating inorganic phosphate by the method of Barlett (10). Cholesterol in these membrane lipid extracts were determined by the method of Courchaine *et al.* (11).

Cholesterol depletion in the basolateral membranes were carried out as follows. Basolateral membrane (2.5 mg of protein) in 0.5 ml of 10 mM Tris-HCl buffer containing 140 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride was treated with 5 μg of digitonin for 15 min at 22 °C. The membrane collected by centrifugation was washed three times with 2 ml of Tris-buffered saline and resuspended in 500 μl of the same buffer. Cholesterol and phospholipid levels were estimated in the lipid extract of digitonin treated membranes as described above.

Cholesterol enrichment of the microsomal membranes was carried out as follows. Cholesterol (25 μg) in chloroform was dried under nitrogen. Microsomal membrane (1 mg of protein) in 1 ml of Tris-HCl buffer was added, vortexed, and incubated for 1 h at either 37 or 5 °C. The membrane thus treated was collected by centrifugation, washed, and resuspended in the same buffer. In some experiments, cholesterol was replaced by its analogues, dihydrocholesterol, and 7-ketocholesterol. Phospholipid and cholesterol (or sterol) levels in these membranes were determined as before.

Phospholipase digestion of basolateral membranes was carried out as follows. Basolateral membranes (300 μg) in 50 μl were digested with phospholipase $A_2$ (15 units, 30 min), phospholipase C (7.5 units, 240 min), or phospholipase D (13 units, 30 min).

*Reconstitution of Pure TC II-R in Synthetic PC and Native Membrane Lipid Vesicles*—Liposomes were prepared using egg PC (2 μmol) alone or egg PC mixed with cholesterol (molar ratio, 1:0.01–1:0.5) and TC II-R (1 μg) by the cholate dialysis method of Low and Zilversmit (12) as described recently (4). Reconstitution of pure TC II-R with DMPC, DPPC, and DSPC vesicles was carried out using a similar protein to PC ratio and a PC to cholesterol ratio of 2:1. The reconstitution experiments were carried out at 5 and 37 °C, 22 and 45 °C, and 22 and 65 °C when the PC used were DMPC, DPPC, and DSPC, respectively. The dialysis and ultracentrifugation of all the samples were performed at 22 °C. After dialysis the liposomally bound TC II-R was collected by centrifugation at 150,000 × *g* for 2 h. Liposomes were also prepared using pure TC II-R, and the lipid was extracted from the native rat microsomal and basolateral membranes isolated using chloroform: methanol mixture (2:1). In each case the total phospholipid used was 2 μmol, which corresponded to a phospholipid/cholesterol (molar ratio) of 26:1 and 1.6:1 in the microsomal and basolateral membrane lipid extracts, respectively. In some experiments, prior to reconstitution, pure TC II-R was digested with *N*-glycosidase or with sialidase followed by treatment with *O*-glycosidase as follows. Pure TC II-R (10 μg) was precipitated with 75% ice-cold ethanol. The pellet obtained was resuspended in 50 μl of 0.075 M sodium phosphate buffer, pH 8.6, containing 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.05% SDS. The reaction mixture was heated at 95 °C for 3 min and cooled. *N*-Octylglucoside (0.6%) and 1,10-phenanthroline (1 mM) were added, and the mixture was incubated for 16 h at 37 °C with PNGase F (0.6 units). Pure TC II-R (10 μg) was digested with sialidase (5 milliunits) for 2 h at 37 °C, and the reaction was stopped by heating the sample for 3 min at 95 °C. The sample was cooled, and SDS (0.1%) was added and further incubated with *O*-glycanase (25 milliunits) for 12 h at 37 °C. The reaction mixture was dialyzed for 2 h, and the dialyzed sample was used for reconstitution.

*Immunoblot Analysis of TC II-R Monomer and Dimer Bound to Native Membranes or Lipid Vesicles*—Immunoblot analysis were carried out to detect the monomer and dimer forms of TC II-R present in the native membranes as follows. Native intestinal total membranes (100–300 μg protein) were subjected to nonreducing SDS-PAGE (7.5%) according to Lammeli (13). The separated proteins were transferred at 90 V to nitrocellulose membranes for various time intervals (15–90 min). The filters were probed with 1000-fold diluted TC II-R antiserum and $^{125}$I-protein A. Protein A was iodinated as described earlier (4, 5) to a specific activity of 10–12 μCi/μg. The bands were visualized by autoradiography and quantified by Ambis radio imaging system. A standard immunoblot was carried out using pure TC II-R, and the blot was quantified and found to be linear in the range 10–1600 ng of TC II-R with corresponding radioimage intensity between 0.006–1 × $10^6$ counts or arbitrary units of 0.3–50 (1 unit = 20,000 counts). When the basolateral (5 μg) or microsomal (50 μg) membranes or lipid vesicle bound TC II-R (25–100 ng) were subjected to immunoblot analysis, the transfer to nitrocellulose membranes was carried out for either 45 or 90 min to detect optimal amounts of the monomer or the dimer species, respectively, present in these membranes (4). Immunoblotting experiments using either native membranes or lipid vesicles were carried out at least three times to confirm the results, particularly the relative mobilities of the two species of TC II-R. In all immunoblotting experiments, the distance between the dimer and the monomer forms of TC II-R was measured and was found to vary by less than 5%.

*Electron Paramagnetic Resonance (EPR) Spectroscopy*—EPR spectroscopy was performed on a Varian Century Series E-109 spectrometer (Varian Associates Inc., Palo Alto, CA) equipped with a $TE_{011}$ rectangular cavity. Samples were contained in a quartz flat cell (Wilmad, Buena, NJ). Data acquisition was controlled by a PC utilizing the VIKING software package (C. C. Felix, Biophysics Research Institute, Milwaukee, WI). Spectra were obtained using 10 mW incident microwave power and a 100 kHz magnetic field modulation of 0.32 Gauss. Field modulation and sweep widths were calibrated with Fremy's salt (potassium nitrosodisulfonate, Aldrich).

## RESULTS

*Immunoblot Analysis of TC II-R Monomer and Dimer Forms*—To develop a convenient method for separating the TC II-R monomer and dimer forms, we first utilized the rather unusual property of TC II-R to stay as dimer when treated with SDS (4). However, in order to optimize the conditions for the recovery of the monomer and dimer forms of TC II-R during immunoblotting, the proteins separated on SDS-PAGE were transferred to nitrocellulose membranes for 15–90 min (Fig. 1). Under identical conditions of electrophoresis and time of exposure to x-ray film, the times required for the optimal transfer of the dimer and the monomer forms were 90 (Fig. 1*A*, *top*) and 45 min (Fig. 1*A*, *bottom*), respectively. However, in order to examine whether both forms of TC II-R can be detected together in a single gel, a transfer time of 60 min was used for immunoblotting of intestinal mucosal total membrane proteins separated on SDS-PAGE. With a transfer time of 60 min, both the monomer and the dimer forms could be seen in the same gel (Fig. 1*B*). Quantitation of the immunoblots shown in Fig. 1*A* revealed that the dimer to monomer ratio was 8:1, whereas it was 2:1 for the data shown in Fig. 1*B*. These results show that a time frame chosen to detect both the monomer and the dimer in the same gel will not quantitatively reflect the actual amount of each species of TC II-R present in any given membrane fraction. Thus, in all subsequent immunoblot analysis, the transfer times employed were 45 and 90 min to visualize

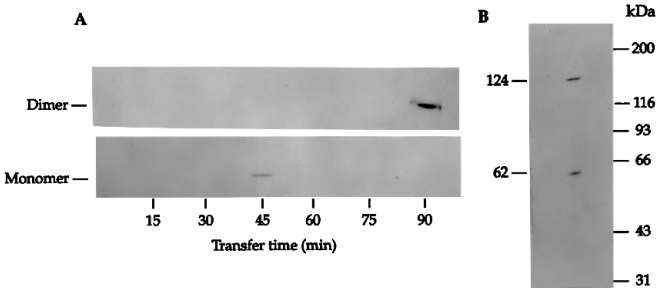


FIG. 1. **Optimization of transfer time during immunoblotting of TC II-R monomer and dimer forms.** *A*, rat intestinal mucosal total membranes (100 $\mu$g) were separated on SDS-PAGE (7.5%). Each lane containing the separated proteins was transferred onto nitrocellulose for the indicated time intervals. The transferred proteins were probed with antiserum to human TC II-R and $^{125}$I-protein A. The *top strip* represents the 124-kDa dimer form, and the *bottom strip* represents the 62-kDa monomer form. The time of exposure to detect both the dimer and monomer forms was the same (18 h). *B*, rat intestinal total membranes (300 $\mu$g) were separated on SDS-PAGE (7.5%). The separated proteins were transferred to nitrocellulose for 60 min and probed as before. The time of exposure was 80 h. Both SDS-PAGE and the immunoblotting were carried out in triplicate, and the same results were obtained each time.

the monomer and the dimer forms of TC II-R, respectively.

*Interactions of Pure TC II-R with Egg PC Vesicles: Effect of Cholesterol Concentration*—Pure TC II-R of molecular mass of 62 kDa was reconstituted into egg PC liposomes containing increasing mol % of cholesterol to study the effect of cholesterol on the lipid vesicle association of TC II-R and its molecular mass following its association with these lipid vesicles. At 22 °C in the absence of added cholesterol, there was no association of TC II-R activity with egg PC lipid bilayer. However, with the increase in the cholesterol content of the liposomes from 1 to 50 mol %, there was an increase from 25 to 80% of receptor activity associated with the lipid bilayer and as a consequence, TC II-R activity in the supernatant fraction decreased by similar amounts (Fig. 2*a*).

Upon SDS-PAGE and immunoblotting of the liposomally bound TC II-R (Fig. 3), the monomer form of 62 kDa was associated with the liposomes prepared with using 1 (Fig. 3*a*, *lane 2*) or 2 mol % (Fig. 3*a*, *lane 1*) of cholesterol (Fig. 3*a*) but not with the liposomes prepared using >10 mol % of cholesterol (Fig. 3*a*, *lanes 3–5*). On the other hand, the 124-kDa dimer was present only when the cholesterol content of the egg PC liposomes was ≥10 mol % (Fig. 3*b*, *lanes 3–5*) but not when the mol % of cholesterol was 1 or 2 (Fig. 3*b*, *lanes 1* and *2*). Quantitation of the immunoblots revealed that TC II-R protein association (Fig. 2*b*) increased by about 9-fold with an increase in cholesterol content of the liposomes from 1 to 50 mol %. These results suggested that the association of TC II-R with egg PC bilayers is dependent on the presence of cholesterol and that its optimal binding and dimerization occurred when the mol % of cholesterol was at least 10. In order to further examine the role of cholesterol in the lipid bilayer and its effect on the dimerization of TC II-R, reconstitution experiments were carried out using synthetic symmetrical PCs of different length fatty acyl residues in the presence and the absence of cholesterol and at temperatures above and below their phase transition.

*Interaction of Pure TC II-R with DMPC, DPPC, and DSPC Vesicles: Effect of Cholesterol and Temperature*—Pure TC II-R associated and dimerized (Fig. 4*a*) in the absence of cholesterol at temperatures below the phase transition of DMPC (5 °C, *lane 1*), DPPC and DSPC (22 °C, *lanes 2* and *3*) vesicles. At the same temperatures, in the presence of cholesterol (50 mol %), there was no association of TC II-R with DMPC or DPPC or DSPC vesicles, and no dimer form could be detected (Fig. 4*a*, *lanes 4–6*). However, at temperatures above their phase transition (Fig. 4*b*), the association and dimerization of TC II-R occurred in the presence of 50 mol % of cholesterol with DMPC (37 °C, *lane 1*), DPPC (45 °C, *lane 2*), and DSPC (65 °C, *lane 3*) vesicles but not in the absence of cholesterol (Fig. 4*b*, *lanes 4–6*). In order to further verify whether the presence of either a monomer or a dimer is solely dependent on the membrane cholesterol, further studies were carried out using native mi-

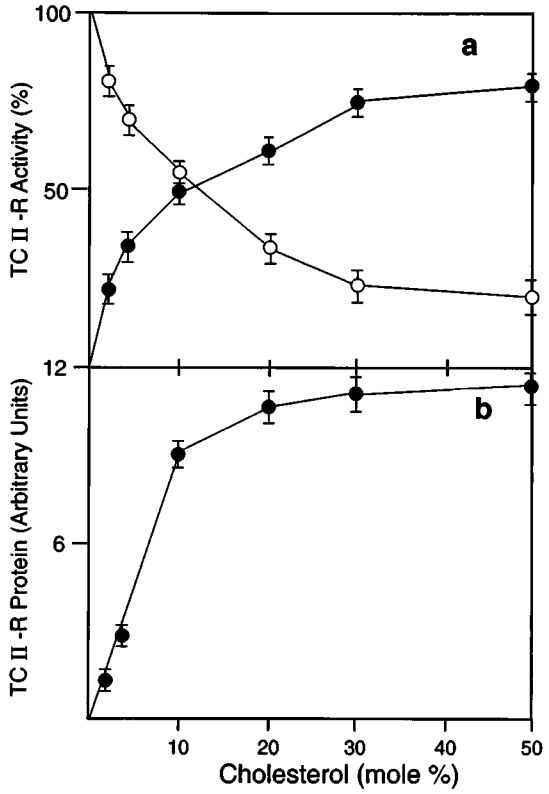


FIG. 2. **TC II-R association with egg PC vesicles containing increasing mol % of cholesterol.** Pure placental TC II-R (1 $\mu$g) was added during the preparation of egg PC vesicles. Cholesterol levels in these vesicles were increased from 0–50 mol %, and the concentration of egg PC used was 2 $\mu$mol. Following dialysis the liposomally bound TC II-R was collected by centrifuging at 150,000 × *g* for 2 h. The supernatant (○) and the lipid pellet (●) were assayed (*a*) for TC II-[$^{57}$Co]Cbl binding. The liposomal pellet was separated on SDS-PAGE and immunoblotted, and the bands were quantified by AMBIS radioimaging system (*b*). One unit of TC II-R associated is equal to 20,000 counts of image density. The reconstitution experiment using various concentration of cholesterol was carried out in triplicates. The results shown represent the means ± S.D. from duplicate assays from each of these reconstitution experiments.

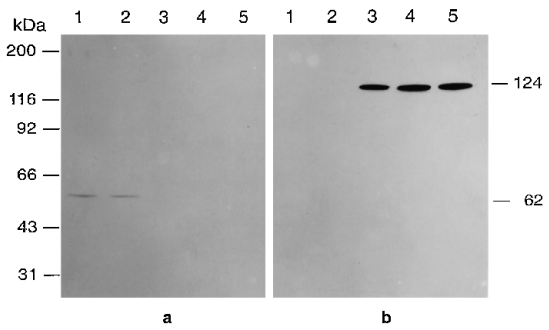


FIG. 3. **Immunoblot analysis of liposomally bound TC II-R.** The liposomal pellet was subjected to SDS-PAGE (7.5%), transferred to nitrocellulose for 45 (*a*) or 90 min (*b*), and probed with diluted antiserum to placental TC II-R and $^{125}$I-protein A. *a*, *lanes 1–5* represent TC II-R associated with egg PC vesicles containing 2, 1, 10, 20, and 50 mol % of cholesterol. *b*, *lanes 1–5* correspond to 1, 2, 10, 20, and 50 mol % of cholesterol.

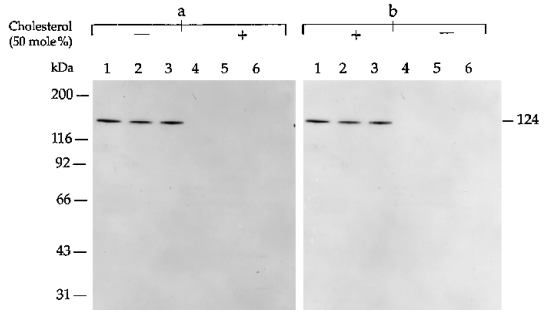


FIG. 4. **Immunoblot analysis of TC II-R associated with DMPC, DPPC, and DSPC vesicles.** *a*, TC II-R bound to DMPC (*lanes 1* and *4*), DPPC (*lanes 2* and *5*), and DSPC (*lanes 3* and *6*) vesicles below their respective transition phase temperatures in the absence (*lanes 1*, *2*, and *3*) and the presence (*lanes 4*, *5*, and *6*) of 50 mol % cholesterol were analyzed on SDS-PAGE (7.5%) and subjected to immunoblot analysis as before. Liposomal preparation were carried out at either 5 °C for DMPC vesicles or room temperature for DPPC and DSPC vesicles. *b*, TC II-R bound to DMPC (*lanes 1* and *4*), DPPC (*lanes 2* and *5*), and DSPC (*lanes 3* and *6*) vesicles above their respective phase transition temperatures in the presence (*lanes 1*, *2*, and *3*) and the absence (*lanes 4*, *5*, and *6*) of 50 mol % cholesterol were analyzed on SDS-PAGE and subjected to immunoblot analysis. Liposomal preparation was carried out at 37, 45, and 65 °C using DMPC, DPPC, and DSPC, respectively.

crosomal and basolateral membranes isolated from rat intestinal mucosa. The use of these membranes were prompted by our recent observation that in many tissue microsomal membranes TC II-R exists as a monomer and that in the basolateral membranes it exists as a dimer (5).

*Reconstitution of Pure TC II-R in Liposomes Prepared Using Lipid Extracts from Native Membranes*—The lipids extracted from the rat intestinal basolateral and microsomal membranes revealed a phospholipid to cholesterol ratio of 1.6:1 and 26:1, respectively. When pure TC II-R was reconstituted with liposomes prepared using 2 μmol of phospholipid in each case, the liposomally bound receptor (Fig. 5, *lane 1*), like in the native microsomes (Fig. 5, *lane 2*), was a monomer. In contrast, TC II-R, when reconstituted with liposomes derived from basolateral membrane lipids, was a dimer (Fig. 5, *lane 7*), like the receptor in the native basolateral membrane (Fig. 5, *lane 8*). TC II-R bound to microsomal lipids vesicles (Fig. 5, *lane 5*) or bound to native microsomal (Fig. 5, *lane 6*) membranes was a monomer and no dimer form of TC II-R could be detected. Similarly, the monomer form of TC II-R could not be detected when TC II-R was bound to basolateral membrane lipid derived liposomes (Fig. 5, *lane 3*) or in the native basolateral membranes (Fig. 5, *lane 4*). These studies again suggested that the dimerization of TC II-R in the native membranes is dependent upon its cholesterol content. In order to directly test this hypothesis, cholesterol levels of these native membranes were altered to determine whether the physical state of TC II-R would change *in situ* without its solubilization from the membranes.

*Effect of Cholesterol Depletion and Enrichment in Native Membranes on the Physical State of TC II-R*—Treatment of the basolateral membranes with digitonin resulted in the depletion of membrane cholesterol altering the phospholipid/cholesterol ratio from 1.6:1 to 27:1. Untreated, cholesterol-depleted, and Triton X-100-treated basolateral membranes were subjected to immunoblot analysis (Fig. 6, *top panel*) and studied for the presence of TC II-R monomer (*panel a*) or the dimer (*panel b*). Native untreated BLM contained only the TC II-R dimer (Fig. 6*b*, *lane 6*) but not the monomer (Fig. 6*a*, *lane 1*). Digitonin-treated BLM contained only the monomer (Fig. 6*a*, *lane 2*), and all the dimer in digitonin-treated membranes was converted to the monomer form (Fig. 6*b*, *lane 7*). The molecular mass of TC II-R monomer formed by digitonin treatment of the BLM was

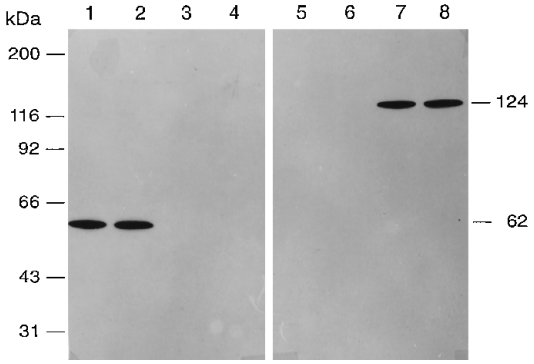


FIG. 5. **Immunoblot analysis of TC II-R bound to liposomes prepared using microsomal and plasma membrane lipids.** Pure TC II-R (25 ng) bound to liposomes prepared using lipid extracts from microsomal (*lane 1* and *5*), basolateral (*lanes 3* and *7*) membrane lipids, native microsomal membrane (50 μg of protein, *lanes 2* and *6*), and basolateral membranes (5 μg of protein, *lanes 4* and *8*) were subjected to SDS-PAGE and immunoblotted as before.

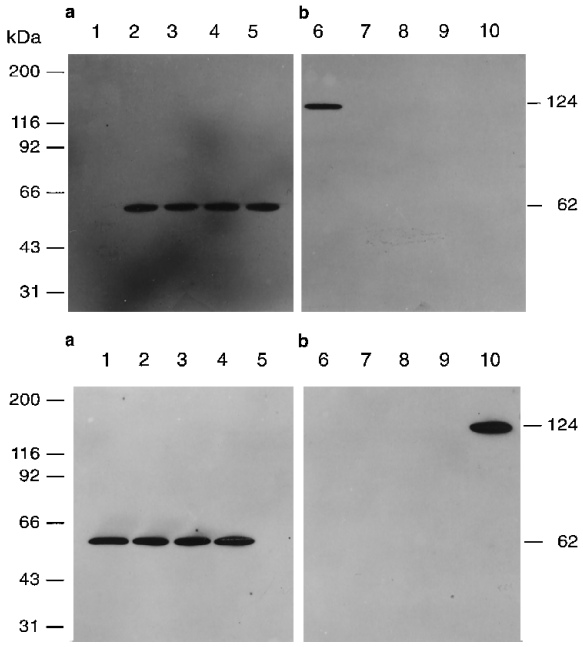


FIG. 6. **Immunoblot analysis of TC II-R monomer and dimer in cholesterol depleted basolateral (*top panel*) and cholesterol enriched microsomal (*bottom panel*) membranes.** *Top panel*, immunoblot analysis of BLM (5 μg of protein) untreated (*lanes 1* and *6*), digitonin-treated (*lanes 2* and *7*), or Triton X-100-treated (*lanes 4* and *9*) or untreated microsomal membranes (50 μg of protein, *lanes 3* and *8*) or Triton X-100-treated microsomes (*lanes 5* and *10*). *Bottom panel*, immunoblot analysis of microsomal membranes, untreated (*lanes 1* and *6*), treated with cholesterol at 5 °C (*lanes 2* and *7*) or treated at 37 °C with dihydrocholesterol (*lanes 3* and *8*), 7-ketocholesterol (*lanes 4* and *9*), and cholesterol (*lanes 5* and *10*). The transfer time during immunoblotting in both *top* and *bottom panels* was either 45 (*a*) or 90 min (*b*).

62 kDa, exactly the same size as the TC II-R monomer present in the native microsomal membrane (Fig. 6*a*, *lane 3*) or the TC II-R present in the Triton X-100 solubilized extract from the BLM (Fig. 6*a*, *lane 4*) or microsomes (Fig. 6*a*, *lane 5*). The absence of TC II-R dimer in native microsomal membranes or Triton X-100-treated BLM and microsomes are shown in Fig. 6*b*, *lanes 8*, *9*, and *10*, respectively.

In contrast to the *in situ* conversion of the TC II-R dimer to the monomer form upon cholesterol depletion of the basolateral membranes, immunoblot analysis (Fig. 6, *bottom panel*) of the microsomal membranes enriched in cholesterol resulted in the conversion of the monomer form (Fig. 6*a*, *lane 1*) to the dimer form (Fig. 6*b*, *lane 10*). The conversion in the physical state of

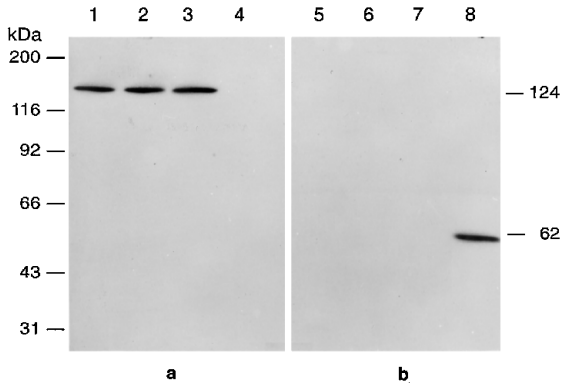


FIG. 7. **Effect of phospholipase $A_2$, C, and D treatment on the molecular mass of basolateral membrane TC II-R.** Untreated BLM (10 $\mu$g) (*lanes 1* and *5*) or digested with phospholipase C (*lanes 2* and *6*), phospholipase D (*lanes 3* and *7*), or phospholipase $A_2$ (*lanes 4* and *8*) were subjected to SDS-PAGE (7.5%), and the separated proteins blotted onto nitrocellulose for 90 (*a*) or 45 min (*b*) were probed with diluted antiserum to human TC II-R and $^{125}$I-protein A. The bands were visualized by autoradiography.

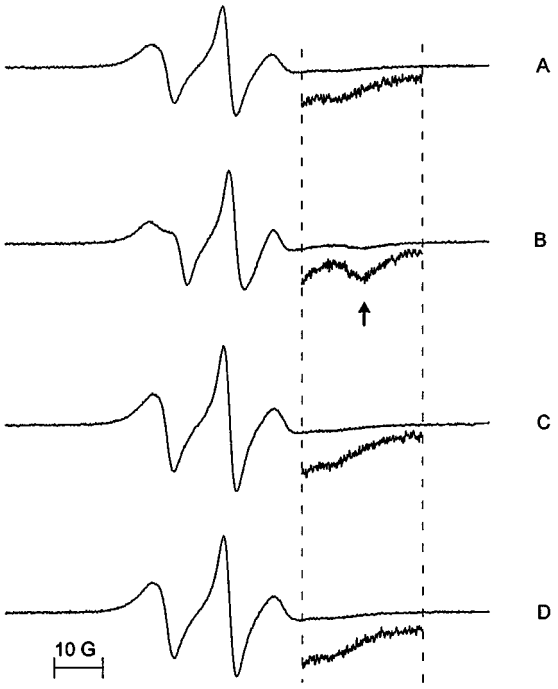


FIG. 8. **EPR spectra of 12PCSL in liposomes prepared from basolateral membrane lipids (*A* and *B*) or microsomal membrane lipids (*C* and *D*).** Samples *B* and *D* contained TC II-R (lipid/protein,3000:1), while samples *A* and *C* contained lipid only. The spin label concentration was 1 mol % relative to total lipids. Spectra were obtained at room temperature (22 °C) and are the average of eight scans. The total scan width was 100 Gauss. Inset beneath each spectrum is a 5× amplification of the high field region emphasizing the strongly immobilized component (*arrow* in *B*) observed only in membranes containing dimeric TC II-R.

TC II-R from the monomer to the dimer form occurred only when the microsomal membranes were incubated with cholesterol at 37 °C (Fig. 6*b*, *lane 10*) but not at 5 °C (Fig. 6*b*, *lane 7*). The specificity of cholesterol effect on the *in situ* conversion of the TC II-R monomer to the dimer form in the microsomal membrane is borne out by the observation that treatment of microsomal membranes with cholesterol analogues such as either dihydrocholesterol (Fig. 6, *a*, *lane 3*, and *b*, *lane 8*) or 7-ketocholesterol (Fig. 6, *a*, *lane 4*, and *b*, *lane 9*) had no effect on the physical state of TC II-R. Following treatment of the microsomal membranes with these analogues, TC II-R remained as a monomer (Fig. 6*a*, *lanes 3* and *4*) without conversion to the dimer form (Fig. 6*b*, *lanes 8* and *9*). However, the treatment of microsomal membranes altered the phospholipid to cholesterol (or sterol) ratio from 26:1 in the native membranes to 18:1, 10.2:1, 11.33:1 and 9.8:1 following treatment of microsomal membranes with cholesterol at 5 °C or at 37 °C or with dihydrocholesterol or 7-ketocholesterol at 37 °C, respectively. Taken together, the results from both the native membranes and synthetic PC bilayers indicated that the association and dimerization of TC II-R may be due to phospholipid-cholesterol interactions, a factor that influences the fluidity (order) of these membranes.

*Importance of Fatty Acyl Resides in Membrane Interactions and Dimerization of TC II-R*—In order to explore the possibility that the cholesterol effects on TC II-R dimerization may be mediated by its interaction with the fatty acyl residue of phospholipid, the following experiments were carried out. When the native basolateral membranes were digested with various phospholipases (Fig. 7), a total *in situ* conversion of the dimer form of TC II-R (Fig. 7*a*, *lane 1*) to the monomer form occurred following treatment with phospholipase $A_2$ (Fig. 7, *a*, *lane 4*, and *b*, *lane 8*) but not phospholipase C (Fig. 7, *a*, *lane 2*, and *b*, *lane 6*) or phospholipase D (Fig. 7, *a*, *lane 3*, and *b*, *lane 7*). Direct evidence for the interaction of TC II-R with the 2-fatty acyl residue was obtained with spin label studies (Fig. 8).

EPR spectra of the phospholipid spin label, 12PCSL, in liposomes prepared from either basolateral or microsomal lipids with and without TC II-R are shown in Fig. 8. Motion of the spin label was significantly restricted in basolateral liposomes containing TC II-R (Fig. 8*B*) relative to the other three systems, as indicated by the increased line widths and appearance of a strongly immobilized high field component in the EPR spectrum. Given the low protein content (protein/lipid, 1:3000) in the reconstituted system, this strongly suggests direct interaction of the dimeric receptor with the *sn*-2-acyl chain of PC that bears the nitroxide label. No such interaction was observed in TC II-R-containing liposomes composed of microsomal lipids, where the receptor is monomeric (Fig. 8*D*). The immobilization was not due to differences in the lipid phase alone, because the rotational motion of the spin label remained fast in both basolateral and microsomal lipid membranes lacking TC II-R (Fig. 8, *A* and *C*). Similar effects were observed in purely model systems and correlated well with dimer formation as observed by SDS-PAGE (*e.g.*, Figs. 4 and 5), where the EPR spectrum of 12PCSL in DMPC/cholesterol liposomes (4:1) at 37 °C, containing TC II-R was significantly broadened relative to that in DMPC/cholesterol (4:1) alone at 37 °C or to that of DMPC vesicles prepared at 37 °C with or without TC II-R (at 37 °C) (data not shown).

*Lack of Effect of TC II-R Glycosylation on Its Dimerization*—The post-microsomal dimerization of TC II-R may be facilitated by the folding alterations due to maturation of its glycan chain(s) during its post-microsomal trafficking. In order to determine the extent to which, if at all, the glycan chains influence folding of TC II-R and thus its dimerization enzymatically deglycosylated mature TC II-R was inserted into egg PC/cholesterol vesicles. The results show mature TC II-R (Fig. 9, *lane 1*) or TC II-R treated with *N*-glycanase (Fig. 9, *lane 2*) when inserted in egg PC/cholesterol (4:1) liposomes demonstrated a molecular mass of 124 kDa. It was difficult to detect the size difference of 2 kDa due to the removal of a single *N*-linked sugar chain. However, upon treatment with sialidase and *O*-glycanase (*lane 3*) or with *N*-glycanase followed by sialidase and *O*-glycanase (*lane 4*), the receptor demonstrated a molecular mass of around 95 kDa, a size that would be predicted for the molecular mass of a dimer formed from a fully

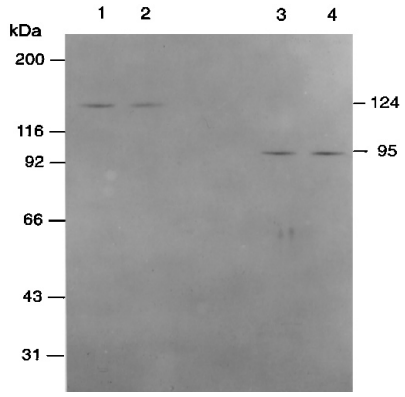


FIG. 9. **SDS-PAGE analysis of enzymatically deglycosylated TC II-R bound to egg PC/cholesterol liposomes.** Egg PC/cholesterol vesicle bound untreated (*lane 1*), *N*-glycanase-treated (*lane 2*), sialidase and *O*-glycanase treated (*lane 3*), and *N*-glycanase, sialidase, and *O*-glycanase treated (*lane 4*) TC II-R (2 $\mu$g) was subjected to SDS-PAGE (7.5%), and the protein bands were visualized following staining of the gel with silver nitrate.

deglycosylated monomer of molecular mass of around 45–47 kDa.

## DISCUSSION

In previous studies (4–6), an immunoblotting procedure to detect and quantify the monomer and the dimer forms of TC II-R was used. This method used a transfer time of 45 min to detect the monomer and 90 min to detect the dimer, respectively. In the current studies, the time of transfer used for the optimal transfer of the monomer and the dimer form has been validated (Fig. 1*A*). The data shown in Fig. 1*A* when quantified demonstrated a monomer to dimer ratio of 1:8, a value similar to that obtained earlier for the distribution of TC II-R monomer and dimer forms in several rat tissue total membranes (5). A single transfer time of 60 min (Fig. 1*B*) can be used to detect both the species of TC II-R in a single gel. However, it will not accurately reflect the absolute steady state amounts of the two species present in any given membrane or the interconversion of one form to the other during experimental modulation of the membranes. When the data shown in Fig. 1*B* was quantified, the ratio of monomer to dimer was 1:2. This value is different than the value of 1:8 obtained using two different transfer times, 45 min to transfer the monomer and 90 min to transfer the dimer. The lower ratio reflects only partial transfer of the dimer and a partial loss of the monomer during the 60-min transfer of the protein from the gel. The decreased transfer of the dimer could be visualized during the 60-min transfer of pure TC II-R bound to PC/cholesterol vesicles by staining the gel for protein. The loss of TC II-R monomer during the 60-min transfer could also be visualized when pure TC II-R was immunoblotted onto a second nitrocellulose filter (data not shown). The likely explanation for the 2-fold time differential for the optimal transfer from SDS-PAGE of the two forms of TC II-R to the nitrocellulose membranes could be that this phenomena is related to the receptor status, *i.e.*, a monomer or a dimer.

In the present work we have addressed issues related to the mechanism of TC II-R dimerization in phospholipid bilayers and in native membranes. Previously (4) we have shown that in native plasma membranes, TC II-R that exists as a dimer of molecular mass of 124 kDa could be converted to a 62-kDa monomer with solubilization of the receptor by Triton X-100 or without its solubilization by delipidation of the plasma membranes with 2:1 mixture of chloroform and methanol. These results clearly indicated that the dimerization of TC II-R is mediated by its strong interactions with the membrane lipids. Some insight into nature of the membrane that may mediate the dimerization of TC II-R was obtained from further studies (5) aimed at understanding the intracellular distribution of TC II-R in the rat kidney. These studies (5) demonstrated that under identical conditions of immunoblotting (except the transfer time), the monomer form of TC II-R with a molecular mass of 62 kDa was detected only in the microsomal membranes, whereas the dimer form of TC II-R with a molecular mass of 124 kDa was the only species present in the plasma membranes. In addition, these studies showed that in many rat tissues, the dimer form of TC II-R was the predominant form present.

One major difference between the plasma and microsomal membranes of many mammalian tissues is the molar ratio of phospholipid to cholesterol. In the plasma membranes the ratio is about 1–1.2:1 (14, 15), whereas it is about 22:1, 20:1, and 26:1 in the microsomal membranes isolated from rat kidney, placenta, and intestinal mucosa, respectively.[2] Because TC II-R exists in two different physical states in these two membranes, we hypothesized that TC II-R dimerization in the native plasma membranes may be related to the presence of higher amount of cholesterol in these membranes. This hypothesis was further based on our earlier observation that TC II-R is able to dimerize with egg PC vesicles that contained 25 mol % of cholesterol (4). In order to explore this hypothesis, additional studies were carried out using both phospholipid vesicles and native microsomal and plasma membranes isolated from rat intestinal mucosa.

Based on the experimental evidence from the current study, we conclude that the single most important factor affecting the dimerization of TC II-R in native plasma membranes and lack of dimerization in the microsomal membranes is the difference in their relative cholesterol content. This conclusion is based on the three lines of evidence. First, the association (Fig. 2) and the ensuing dimerization of TC II-R with egg PC occurred with a minimum cholesterol content of 10 mol % (Fig. 2). At cholesterol levels of 1 or 2 mol %, TC II-R associated poorly with egg PC vesicles and stayed as a monomer. Second, mature TC II-R dimerized upon insertion into lipid vesicles prepared using total lipids from intestinal basolateral but not microsomal membranes (Fig. 5). The phospholipid to cholesterol ratios in these extracts were 1.2:1 and 26:1, respectively (data not shown). Third, changing the phospholipid/cholesterol ratio from 1.2:1 to 26:1 in the basolateral plasma membranes by cholesterol depletion with digitonin treatment (Fig. 6, *top panel*) or from 26:1 to 10:1 in the microsomal membranes by cholesterol enrichment (Fig. 6, *bottom panel*) resulted in the *in situ* conversion of the dimer form to the monomer form and from the monomer form to the dimer form, respectively.

How does cholesterol influence the interaction between the two monomers of TC II-R in order to facilitate the formation of noncovalent dimers in the native membranes? The answer may be related to the potential function(s) of cholesterol in membranes. Cholesterol could play a role in facilitating the hydrophobic match between the lipid bilayer and TC II-R, thus leading to its association and dimerization. Hydrophobic matching is a well established model for lipid-protein interactions in the membranes (16–18). This model states that in order to accommodate or match the hydrophobic region of a protein with that of the hydrophobic core of lipid bilayer, association of a protein is accompanied by alterations in the thickness of lipid bilayers. Earlier studies (19–21) have confirmed that physiological membrane functions are controlled by hydrophobic membrane thickness. For example, optimal activity of

[2] S. Bose and B. Seetharam, unpublished observations.

$Ca^{2+}$-ATPase (22, 23) and acetylcholine receptor (24, 25) function can be altered by the introduction of *n*-alkanes, which are known to increase hydrophobic thickness of the lipid bilayer. Like *n*-alkanes, cholesterol is also known to increase the hydrophobic thickness of lipid bilayers (26, 27). Therefore one possible explanation for the cholesterol induced effects of TC II-R association and the ensuing dimerization could be that it is due to the cholesterol-mediated thickening of lipid bilayer. During such a thickening, hydrophobic matching between TC II-R and the hydrophobic core of the membrane could facilitate two monomers of TC II-R to be spatially near to one another and dimerize encompassing a specific lipid milieu within the bilayer.

Based on experimental evidence, a more likely possible role of cholesterol in the dimerization of TC II-R is that it is due to interaction of cholesterol with the phospholipid. X-ray and neutron scattering studies have shown that cholesterol inserts normal to the plane of the bilayer with the β-OH group near the ester carbonyl of the lipid (28, 29). It is thought that this results in the development of hydrogen bonding between the β-hydroxyl group of cholesterol and the carbonyl oxygen linking the fatty acyl chains with its glycerol backbone (30). However, Raman spectroscopy indicates that no actual hydrogen bonding occurs with these carbonyls (31). Despite this uncertainty, what is generally agreed upon is that cholesterol has a substantial effect on the order parameters measured along the lipid hydrocarbon chain by $^2$H-NMR (32) and on the phase transition of the phospholipid (33). Fourier transform infrared spectroscopy studies (34) have shown that above the transition midpoint cholesterol decreases the fraction of *gauche* rotomers in the phospholipid hydrocarbon chain, whereas just the opposite is the effect below the transition midpoint.

Effect of cholesterol on the membrane interactions of TC II-R are mediated by increasing the order around the fatty acyl residues. Several lines of evidence support that the order around the 2-fatty acyl residue is important for the interactions of TC II-R with lipid bilayers. One, cholesterol analogues were unable to mediate the dimerization of TC II-R in the fluid microsomal membrane (Fig. 6), suggesting very strongly that the cholesterol-induced dimerization of TC II-R is due to increased interaction of the highly hydrophobic TC II-R with a rigid microdomain of the bilayer. Thus, with only 4% cholesterol, a more fluid microsomal membrane will not support TC II-R dimerization, as opposed to the basolateral membrane, a more ordered (less fluid) membrane with nearly 40% cholesterol. Two, the ability of phospholipase $A_2$ but not other phospholipases to convert *in situ*, the TC II-R dimer to the monomer form and the ESR studies support the concept that TC II-R dimerization is due to phospholipid fatty acyl chain-cholesterol interaction. Three, the role of cholesterol in increasing the rigidity of phospholipid bilayers above their transition temperature is well accepted (35–37). The results obtained with DMPC, DPPC, and DSPC show that cholesterol-induced dimerization occurred above but not below the transition temperature of these PCs, once again suggesting that increased association and the ensuing dimerization of TC II-R is due to cholesterol-mediated increase of bilayer rigidity/molecular order or decreased fluidity. It is interesting to note that the addition of 50 mol % cholesterol to these vesicles below their transition temperature resulted in very poor or no binding of TC II-R, clearly indicating that disappearance of phase transition (38) is not conducive for the association and dimerization of TC II-R.

One of the important regulators of intracellular folding of proteins is its post-translational modifications. With respect to TC II-R, these include maturation of a single *N*-linked oligosaccharide to the complex type and the maturation of its *O*-linked sugars. TC II-R contains several *O*-linked sugar residues, and the contribution of its molecular mass of 62 kDa by the *O*-linked sugars is about 15 kDa and by *N*-linked sugars is about 2 kDa. Due to the presence of at least 27–28% of sugars in TC II-R, it is likely that they may play a role in influencing the folding of TC II-R required for its interactions with the lipid bilayer and the ensuing dimerization. The results (Fig. 9) convincingly show that the dimerization of TC II-R is independent of the potential folding alterations of the receptor during its carbohydrate maturation and intracellular trafficking. Other potential co- and post-translational modifications of TC II-R that could play a role in its interactions with the lipid bilayer and influence its dimerization include different types of covalent lipid modification. TC II-R expressed in either intestinal-derived Caco-2 cells or proximal tubular-derived opossum kidney cells when labeled with [$^3$H]myristate or palmitate or mevelonate failed to incorporate the label into TC II-R, although other proteins expressed in these cell lines were labeled with one or the other of these labels. In addition, incubation of Caco-2 cells with cerulenin, an inhibitor of palmitoylation of proteins (39) failed to inhibit either the surface domain expression or the $T_{½}$ of TC II-R, suggesting that TC II-R is not palmitoylated.[2] Taken together these observations strongly argue for the highly ordered lipid bilayer microenvironment as the sole determinant of hydrophobic TC II-R monomers to dimerize in tissue plasma membranes.

In conclusion, the results of the present study have shown that the dimerization of TC II-R, an important nutrient receptor, is a post-microsomal event and is due to its strong interactions with a highly ordered lipid bilayer membrane. The membrane fluidity regulated dimerization of plasma membrane TC II-R noted in this study may represent an unique situation. Other factors such as ligand binding (40) and phosphorylation-dephosphorylation (41) are known to regulate the oligomerization of several receptors. Finally, it is not known what physiological advantage a cell might have with TC II-R dimers in their plasma membranes. However, because the dimers are functional in ligand binding and bind 2 mol of ligand/dimer, it is likely that the existence of dimers will help in the rapid uptake of circulatory Cbl. Further studies are needed to identify the hydrophobic regions of TC II-R that mediate its interaction with the lipid bilayer, and such studies are possible once the sequence of TC II-R is known.

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